An Antibiotic Detected in Conifer Foliage and Its Relation to Cenococcum graniforme Mycorrhizae

Note by L. F. Grand and W. W. Ward

Abstract. *Cenococcum graniforme* formed abundant mycorrhizae with *Pinus strobus*, *P. resinosa*, and *Picea abies* growing in pure plantations on two soil types in central Pennsylvania. Roots of these species growing in Morrison sandy loam had more *C. graniforme* mycorrhizae than those in Calvin-Edom silty clay loam. *Picea abies* showed the greatest number of mycorrhizae per unit length of root and *Pinus strobus* the fewest. An antibiotic similar to that produced by *C. graniforme* in vitro was detected in needles of each species. Bioassay of the *Cenococcum* antibiotic with *Bacillus cereus* indicated a significantly greater antibiotic activity in *P. strobus* than in *P. resinosa*. *Picea abies* extracts contained the antibiotic but apparently not in sufficient quantity to inhibit *B. cereus*.

Additional key words. *Pinus strobus*, *Pinus resinosa*, *Picea abies*.

Several mechanisms by which mycorrhizal fungi may serve as protectants against soil-borne pathogens have been postulated (Zak 1964). Santoro and Casida (1962), who have identified antibiotics produced by *Suillus luteus* (Fr.) S. F. Gray and several other mycorrhizal fungi, suggest that the antibiotics may serve as effective barriers against attack by soil-inhabiting pathogens. Ectotrophic mycorrhizae of pine have been demonstrated to be antagonistic to root pathogenic fungi and soil bacteria and effective in providing resistance to infection by *Phytophthora cinnamoni* Rands (Marx 1969a; Marx and Davey 1969a, b). Mycorrhizae formed by *Leucopaxillus cerealis* (Lasch) Sing. var. *piceina* with *Pinus echinata* Mill. were resistant to infection by *P. cinnamoni* (Marx and Davey 1969a). The fungus produced antibiotics in pure culture (Marx 1969b).

An antibiotic produced in pure cultures of *Cenococcum graniforme* (Sow.) Ferd. and Winge was isolated (Krywolap and Casida 1964) and also shown to be present in roots and foliage of eastern white pine (*Pinus strobus* L.), red pine (*P. resinosus* Ait.), and Norway spruce [*Picea abies* (L.) Karst.] bearing mycorrhizae formed by this fungus (Krywolap *et al.* 1964).

Little is known about the natural occurrence of the mycorrhizae of *C. graniforme* on conifers in eastern United States or about the production of the antibiotic under forest conditions. This study was initiated to determine (1) the abundance of mycorrhizae on roots of white pine, red pine, and Norway spruce in relation to soil type and site quality, and (2) the relationship between the abundance of mycorrhizae and the presence of the antibiotic in the foliage.

Methods and Procedures

Pure plantations of each species were selected on two soil types in central Pennsylvania—Morrison sandy loam and Calvin-Edom silty clay loam. Two plots were established on each soil type: one on a good site (dominant trees 9.7 to 13.7 m in height at 25 years of age) and the other on a poorer site (dominant trees 7.6 to 9.7 m in height at 25 years of age).

Three samples of roots and soil were obtained near each of three trees on each plot. The recent litter was removed and a core 5 cm in diameter was taken to a 5 cm depth. Adhering soil was gently washed from the roots by placing the core in flowing tap water for 10 to 25 min. Segments of roots were selected at random, and the numbers of *C. graniforme* mycorrhizae per cm of root length recorded.

Foliage from the current year's growth was collected from the upper and middle portions of the live crown and stored at −20°C until extraction. Antibiotic substances were extracted according to the procedure of Krywolap and Casida (1964). Fifteen grams of foliage were homogenized in a Waring Blendor with 150 ml of acetone for 5 minutes. The extract was filtered twice through Whatman No. 1 paper and evaporated to 10 ml. A qualitative bioassay of the extract was made, with

---

The authors are, respectively, Asst. Professor of Plant Pathology, School of Agric. and Life Sciences, N. Car. State Univ. (formerly Research Assistant at the School of Natural Resources, Pa. State Univ.); and Professor of Silviculture, School of Natural Resources, Pa. State Univ. This Paper was authorized for publication as Paper No. 2990 in the journal series of Pa. Agric. Expt. Sta. Manuscript accepted Dec. 8, 1968.

Bacillus cereus Cohn as the test organism. Inhibiting extracts were subjected to 2-dimensional ascending paper chromatography (Krywolap *et al.* 1964) to establish the presence of the "*Cenococcum* antibiotic". Chromatograms were developed in the first dimension in a solution of methanol, ammonium hydroxide, and water (3:3:1 by volume) for approximately 130 minutes. After drying for 24 hours, they were developed in the second dimension in the upper phase of benzene saturated with 10-percent acetic acid for 85 minutes and air-dried again for 24 hours. The antibiotic was identified by Rf values and blue-green fluorescence under short wavelength ultraviolet light (Krywolap and Casida 1964). After isolation a quantitative bioassay for the antibiotic was performed by cutting the spots from the chromatograms and placing them on a 0.1-percent glucose nutrient agar medium inoculated with *B. cereus*. Zones of inhibition were measured following an incubation period of 18 hours at 30°C. The ratio of the area of the inhibition zone to that of the chromatogram spot was taken as a measure of the relative amount of antibiotic activity.

The significance of differences in numbers of mycorrhizae and antibiotic activity among the tree species, crown position, soil type, and site quality were determined by analysis of variance.

Results and Discussion

More mycorrhizae were found on all species growing on Morrison sandy loam than on Calvin-Edom silty clay loam (3.1 compared to 2.3 per cm of root length for all sites and species taken together, Table 1). More mycorrhizae occurred on Norway spruce (3.5/cm) than on white pine (1.8/cm) or red pine (2.7/cm); the difference between Norway spruce and white pine was significant at the 5-percent level. These results confirm the findings of Trappe[1] that spruces tend to have more mycorrhizae than pines and that more mycorrhizae are formed by *C. graniforme* on droughty soil than on less droughty soils. No significant difference in the numbers of mycorrhizae was found between good and poor sites.

The Rf values (0.69 and 0.00) for chromatographic separation of the *Cenococcum* antibiotic in extracts of foliage from the three species of trees agreed favorably with those reported by Krywolap and Casida (1964) (0.75 and 0.00) for acetone extracts of mycelium grown in culture. The antibiotic spot fluoresced under short wavelength ultraviolet light, emitting a blue-green color similar to that reported by Krywolap and Casida.

Antibiotic activity of foliage extract was significantly greater in white pine than in red pine. Although the antibiotic was detected on chromatograms of Norway spruce extracts, the amount present was not sufficient to inhibit *B. cereus*, the test organism. Activity did not differ significantly between the two crown positions sampled.

There was no direct relationship between the number of *Cenococcum* mycorrhizae and the activity of antibiotic from the foliage of a species (Table 1). White pine, with the fewest number of mycorrhizae per cm of root, had the highest inhibition index. In contrast, Norway spruce, with the greatest number of mycorrhizae, displayed no measurable inhibiting activity.

[1] Trappe, J. M. 1962. *Cenococcum graniforme*—its distribution ecology, mycorrhiza formation and inherent variation. Ph.D. thesis, 176 pp. Univ. Wash., Seattle.

TABLE 1. Abundance of mycorrhizae of Cenococcum graniforme and inhibition activity of "Cenococcum antibiotic" extracted from foliage of three coniferous species.

Species	Site	Number of mycorrhizae per cm of root length			Inhibition index[1]	
		Morrison sandy loam	Calvin–Edom silty clay loam	Average	Morrison sandy loam	Calvin–Edom silty clay loam
White pine	Good	1.7	1.2	1.8	1.81	1.48
	Poor	2.8	1.7		1.68	1.52
Red pine	Good	3.1	2.3	2.7	1.35	1.13
	Poor	3.0	2.5		1.20	1.19
Norway spruce	Good	4.1	2.6	3.5	1.00	1.00
	Poor	4.0	3.2		1.00	1.00

[1] Ratio of the area of the inhibition zone to the area of the antibiotic spot from the chromatogram. Value of 1.00 equals no inhibition of *Bacillus cereus*.

Physiological differences between species of *Picea* and *Pinus* may have resulted in the inactivation of antibiotic activity in the former and not in the latter. No attempt was made to identify different strains of *C. graniforme*, but the presence of the same strain on different hosts would suggest a host influence on the antibiotic, either in its production by the fungus or alteration within the host. Unlike strains of *C. graniforme* may have contributed to the difference in antibiotic activity, but this seems doubtful as Krywolap and Casida (1964) reported similar antibiotic production *in vitro* from seven isolates of *C. graniforme* from Maryland and Northwestern United States.

Inasmuch as trees without *C. graniforme* mycorrhizae could not be found in nature, extracts from trees not infected with this fungus could not be compared with those from infected trees.

Literature Cited

KRYWOLAP, G. N., and L. E. CASIDA, JR. 1964. An antibiotic produced by the mycorrhizal fungus *Cenococcum graniforme*. Can. J. Microbiol. 10: 365–370.

———, L. F. GRAND, and L. E. CASIDA, JR. 1964. Natural occurrence of an antibiotic in the mycorrhizal fungus *Cenococcum graniforme*. Can. J. Microbiol. 10:323–328.

MARX, D. H. 1969a. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology. (*In press.*)

———. 1969b. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by *Leucopaxillus cerealis* var. *piceina*. Phytopathology. (*In press.*)

———, and C. B. DAVEY. 1969a. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. III. Resistance of aseptically formed mycorrhizae to infection by *Phytophthora cinnamoni* Rands. Phytopathology. (*In press.*)

———, and C. B. DAVEY. 1969b. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of naturally occurring mycorrhizae to infections by *Phytophthora cinnamoni* Rands. Phytopathology. (*In press.*)

SANTORO, T., and L. E. CASIDA, JR. 1962. Elaboration of antibiotics by *Boletus luteus* and certain other mycorrhizal fungi. Can. J. Microbiol. 8: 43–48.

ZAK, B. 1964. Role of mycorrhizae in root disease. Ann. Rev. Phytopath. 2:377–392.